

**COMPOSITIONS AND METHODS FOR DELIVERING THYMOSIN BETA 4,  
ANALOGUES, ISOFORMS AND OTHER DERIVATIVES**

**BACKGROUND OF THE INVENTION**

**CROSS-REFERENCE TO RELATED APPLICATION**

**[001]** This application claims the benefit of U.S. Provisional Application No. 60/458,399, filed March 31, 2003.

**Field of the Invention**

**[002]** The present invention relates to the field of compositions and methods for delivering polypeptide pharmaceuticals.

**Description of the Background Art**

**[003]** Polypeptide pharmaceuticals can be extremely efficacious agents in the treatment of various maladies. Since polypeptide pharmaceuticals can be very expensive to produce, there is a need in the art for improved compositions and methods for delivering polypeptide pharmaceuticals.

**SUMMARY OF THE INVENTION**

**[004]** In accordance with the present invention, a composition comprises a substantially purified composition including an adhesive and a polypeptide comprising amino acid sequence LKKTET, or a conservative variant thereof. A method of delivery of a polypeptide to a site comprises introducing the above composition to the site.

## DETAILED DESCRIPTION OF THE INVENTION

**[005]** The present invention provides compositions and methods utilizing actin-sequestering peptides such as thymosin  $\beta$ 4 (T $\beta$ 4) and other actin-sequestering peptides or peptide fragments containing amino acid sequence

5 LKKTET or conservative variants thereof. Included are NB or C-terminal variants such as KLKKTET and LKKTETQ. These peptides and peptide fragments are useful in promoting wound healing and other physiological uses.

**[006]** Thymosin  $\beta$ 4 was initially identified as a protein that is up-regulated during endothelial cell migration and differentiation *in vitro*. Thymosin  $\beta$ 4 is a 43  
10 amino acid, 4.9 kDa ubiquitous polypeptide identified in a variety of tissues. Several roles have been ascribed to this protein including a role in a endothelial cell differentiation and migration, T cell differentiation, actin sequestration and vascularization.

**[007]** Thymosin  $\beta$ 4 is a member of the  $\beta$ -thymosin family of highly conserved  
15 polar 5-kDa polypeptides found in various tissues and cell types. Originally purified from thymus and regarded as a thymic hormone, thymosin  $\beta$ 4 was then found to be involved in multiple biological processes. As the main G-actin sequestering peptide, it plays an important role in regulation of actin assembly during cell proliferation, migration, and differentiation. Numerous studies  
20 implicate thymosin  $\beta$ 4 in regulation of cancerogenesis, inflammation, angiogenesis, and wound healing. It was found that thymosin  $\beta$ 4 expression regulated tumorigenicity and metastatic activity in malignant cell lines through actin-based cytoskeletal organization. Thymosin  $\beta$ 4 was found to be elevated in tube forming endothelial cells; it increases their attachment, spreading and  
25 migration thus promoting angiogenesis. Thymosin  $\beta$ 4 was also found in ulcer

extracts and wound fluids at high concentrations and was suggested to function as an antibacterial factor. The stimulating role of thymosin  $\beta$ 4 in wound healing was demonstrated in several studies with animal models. When added topically or administered intraperitoneally, thymosin  $\beta$ 4 enhanced dermal wound healing in a rat full thickness model. The ability to accelerate dermal wound healing has also been observed in db/db diabetic mice, steroid-immunosuppressed mice and in aged mice. Thymosin  $\beta$ 4 has also been shown to accelerate healing of the corneal epithelium after burn injuries and to down regulate a number of corneal cytokines and chemokines reducing the inflammatory response.

10 [008] Activation of the coagulation cascade upon vascular injury results in generation of thrombin which converts fibrinogen into fibrin. Fibrin polymerizes spontaneously to form blood clots which seals damaged places thus preventing the loss of blood. Fibrin also serves as a provisional matrix on which various cell types adhere, migrate and proliferate replacing fibrin with normal tissues during subsequent wound healing processes. Factor XIIIa, a plasma transglutaminase, covalently cross-links the fibrin clot reinforcing its structure. In addition, it also cross-links to fibrin a number of physiologically active proteins which may modulate properties of the fibrin matrix. For example, covalent incorporation of  $\alpha_2$ -antiplasmin increases resistance of the matrix to fibrinolysis and

15 incorporation of fibronectin may affect its ability to support cell adhesion and migration. Tissue transglutaminase can selectively incorporate into fibrin thymosin  $\beta$ 4.

[009] Thymosin  $\beta$ 4 serves as a specific substrate for tissue transglutaminase and can be selectively cross-linked by it to collagen, actin, fibrinogen and fibrin, proteins which are also involved in the above mentioned processes. After

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activation of platelets with thrombin, thymosin  $\beta$ 4 is released and cross-linked to fibrin in a time- and calcium-dependent manner. Platelet factor XIIIa is co-released from stimulated platelets. Cross-linking of platelet-released thymosin  $\beta$ 4 to fibrin appears to be mediated by factor XIIIa and provides a mechanism to increase the local concentration of thymosin  $\beta$ 4 near sites of clots and tissue damage, for promotion of wound healing, angiogenesis and inflammatory response.

**[0010]** Fibrinogen is a chemical dimer comprising two identical subunits, each composed of three polypeptide chains,  $A\alpha$ ,  $B\beta$  and  $\gamma$  held together by a number of disulfide bonds. The disulfide-linked  $NH_2$ -terminal portions of all six chains form the central E region, while the  $COOH$ -terminal portions form two terminal D regions and two  $\alpha C$ -domains. Upon conversion of fibrinogen into fibrin, thrombin-mediated removal of the  $NH_2$ -terminal fibrinopeptides A and B from the fibrinogen and removal of the  $NH_2$ -terminal fibrinopeptides A and B from the fibrinogen  $A\alpha$  and  $B\beta$  chains, respectively, results in exposure of their active sequences (polymerization sites) and enables interaction between the E and D regions of neighboring molecules (DD:E interaction) to form a fibrin polymer. The polymer becomes cross-linked by factor XIIIa through the  $COOH$ -terminal portions of the fibrin  $\alpha$  and  $\gamma$  chains. The intermolecular cross-linking of the  $\gamma$  chains of the adjacent D regions occurs rapidly resulting in  $\gamma$ - $\gamma$  dimers, while cross-linking between the  $\alpha$  polymers ( $\alpha C$ -domains) occurs more slowly and results in formation of  $\alpha$  polymers. In addition, the  $\alpha$  chains serve for cross-linking to fibrin of such proteins as fibronectin,  $\alpha_2$ -antiplasmin, and PAI-2. Thus, it is tempting to hypothesize that these chains could also be involved in cross-linking of thymosin  $\beta$ 4.

**[0011]** To clarify the mechanism of the incorporation of thymosin  $\beta$ 4 into fibrin(ogen), its interaction was studied with fibrinogen, fibrin and their recombinant fragments (domains) in the absence and presence of factor XIIIa. The study revealed that although there appears to be no substantial non-covalent interaction between fibrin(ogen) and thymosin  $\beta$ 4, factor XIIIa efficiently cross-  
5 links the latter to both fibrinogen and fibrin and that cross-linking occurs mainly through the COOH-terminal portion of their  $\alpha$ C-domains including residues 392-610.

**[0012]** In accordance with one embodiment, a substantially purified  
10 composition is provided which includes an adhesive and a polypeptide comprising amino acid sequence LKKTET or a conservative variant thereof. In accordance with one embodiment, the adhesive is capable of adhering to medical devices such as stents. In a particularly preferred embodiment, the adhesive is capable of adhering to tissues of a living subject such as a human.

**[0013]** In preferred embodiments, the adhesive is a biodegradable adhesive. When used herein, the term biodegradable adhesive is intended to encompass bioabsorbable or errodable adhesives. In preferred embodiments, the invented composition initially is in a fluid or semi-fluid state, most preferably in a liquid or semi-liquid state. In particularly preferred embodiments, after application, the  
20 adhesive increases in viscosity or at least partially solidifies while adhering to the tissue. The composition may be introduced by applying to an area in a layer, most preferably by spraying or with a brush.

**[0014]** In preferred embodiments, the adhesive utilized in the present invention is a fibrin sealant matrix (fibrin glue). Fibrin glue is a two-component system of  
25 separate solutions of fibrinogen and thrombin/calcium. When the two solutions

are combined, the resultant mixture mimics the final stages of the clotting cascade to form a fibrin clot. The fibrinogen component can be prepared extemporaneously from autologous, single-donor, or pooled blood. Fibrin glue is available in Europe under the brand names Beriplast, Tissel, and Tissucol.

5 Fibrin glue has been used in a wide variety of surgical procedures to repair, seal, and attach tissues in a variety of anatomic sites.

[0015] Thus, the present invention provides a method of delivering an LKKTET polypeptide to a site of a living subject. In preferred embodiments, this site is a surface. The inventive method comprises applying the inventive composition to  
10 the site. In preferred embodiments, the site is a wound, such as an acute or chronic wound.

[0016] In preferred embodiments, the adhesive is fibrin, fibrinogen, fibrin glue, a collagen, fragments of any of the above or a mixture of any of the above. Collagen adhesives which may be utilized include types 1, 2, 3, 4 and/or 5  
15 collagens. Other adhesives may include actin or integrin adhesives.

[0017] In other embodiments, the biodegradable adhesive utilized in the inventive composition is a gel (*e.g.*, adhesive collagen gel), gel/fibrin mixture, powder or the like.

[0018] In preferred embodiments, the adhesive is covalently bound to the  
20 LKKTET peptide, most preferably by factor XIIIa. In particularly preferred embodiments, the adhesive is a fragment of fibrin or fibrinogen.

[0019] In preferred embodiments, the LKKTET polypeptide comprises amino acid sequence KLKKTET or LKKTETQ, Thymosin  $\beta$ 4 (T $\beta$ 4), an N-terminal variant of T $\beta$ 4, a C-terminal variant of T $\beta$ 4, an isoform of T $\beta$ 4, a splice-variant of T $\beta$ 4,  
25 oxidized T $\beta$ 4, T $\beta$ 4 sulfoxide, lymphoid T $\beta$ 4, pegylated T $\beta$ 4 or any other actin

sequestering or bundling proteins having actin binding domains, or peptide fragments comprising or consisting essentially of the amino acid sequence LKKTET or conservative variants thereof. International Application Serial No. PCT/US99/17282, incorporated herein by reference, discloses isoforms of T $\beta$ 4

5 which may be useful in accordance with the present invention as well as amino acid sequence LKKTET and conservative variants thereof, which may be utilized with the present invention. International Application Serial No.

PCT/GB99/00833 (WO 99/49883), incorporated herein by reference, discloses oxidized Thymosin  $\beta$ 4 which may be utilized in accordance with the present

10 invention. Although the present invention is described primarily hereinafter with respect to T $\beta$ 4 and T $\beta$ 4 isoforms, it is to be understood that the following description is intended to be equally applicable to amino acid sequence LKKTET, LKKTETQ, peptides and fragments comprising or consisting essentially of LKKTET or LKKTETQ, conservative variants thereof, as well as oxidized Thymosin  
15  $\beta$ 4.

**[0020]** Examples of contacting the damaged site include contacting the site with a composition comprising adhesive/T $\beta$ 4 alone, or in combo with at least one agent that enhances T $\beta$ 4 penetration, or delays or slows release of T $\beta$ 4 peptides into the area to be treated. A subject may be a mammal, preferably human.

20 **[0021]** T $\beta$ 4, or its analogues, isoforms or derivatives, may be administered in any suitable effective amount. For example, T $\beta$ 4 may be administered in dosages within the range of about 0.1-50 micrograms of T $\beta$ 4, more preferably in amounts within the range of about 1-25 micrograms.

**[0022]** A composition in accordance with the present invention can be  
25 administered daily, every other day, etc., with a single administration or multiple

administrations per day of administration, such as applications 2, 3, 4 or more times per day of administration.

[0023] T $\beta$ 4 isoforms have been identified and have about 70%, or about 75%, or about 80% or more homology to the known amino acid sequence of T $\beta$ 4. Such isoforms include, for example, T $\beta$ 4<sup>ala</sup>, T $\beta$ 9, T $\beta$ 10, T $\beta$ 11, T $\beta$ 12, T $\beta$ 13, T $\beta$ 14 and T $\beta$ 15. Similar to T $\beta$ 4, the T $\beta$ 10 and T $\beta$ 15 isoforms, as well as the T $\beta$ 4 splice-variants, have been shown to sequester actin. T $\beta$ 4, T $\beta$ 10 and T $\beta$ 15, as well as these other isoforms share an amino acid sequence, LKKTET, that appears to be involved in mediating actin sequestration or binding. Although not wishing to be bound to any particular theory, the activity of T $\beta$ 4 isoforms may be due, in part, to the ability to regulate the polymerization of actin.  $\beta$ -thymosins appear to depolymerize F-actin by sequestering free G-actin. T $\beta$ 4's ability to modulate actin polymerization may therefore be due to all, or in part, its ability to bind to or sequester actin via the LKKTET sequence. Thus, as with T $\beta$ 4, other proteins which bind or sequester actin, or modulate actin polymerization, including T $\beta$ 4 isoforms having the amino acid sequence LKKTET, are likely to be effective, alone or in a combination with T $\beta$ 4, as set forth herein.

[0024] Thus, it is specifically contemplated that known T $\beta$ 4 isoforms, such as T $\beta$ 4<sup>ala</sup>, T $\beta$ 9, T $\beta$ 10, T $\beta$ 11, T $\beta$ 12, T $\beta$ 13, T $\beta$ 14 and T $\beta$ 15, as well as T $\beta$ 4 isoforms and T $\beta$ 4 splice-variants not yet identified, will be useful in the methods of the invention. As such T $\beta$ 4 isoforms are useful in the methods of the invention, including the methods practiced in a subject. The invention therefore further provides pharmaceutical compositions comprising T $\beta$ 4, as well as T $\beta$ 4 isoforms T $\beta$ 4<sup>ala</sup>, T $\beta$ 9, T $\beta$ 10, T $\beta$ 11, T $\beta$ 12, T $\beta$ 13, T $\beta$ 14 and T $\beta$ 15, and a pharmaceutically acceptable carrier.



**[0025]** In addition, other proteins having actin sequestering or binding capability, or that can mobilize actin or modulate actin polymerization, as demonstrated in an appropriate sequestering, binding, mobilization or polymerization assay, or identified by the presence of an amino acid sequence that mediates actin binding, such as LKKTET, for example, can similarly be employed in the methods of the invention. Such proteins include gelsolin, vitamin D binding protein (DBP), profilin, cofilin, adsevertin, propomyosin, fincilin, depactin, DnaseI, villin, fragmin, severin, capping protein,  $\beta$ -actinin and acumentin, for example. As such methods include those practiced in a subject, the invention further provides pharmaceutical compositions comprising gelsolin, vitamin D binding protein (DBP), profilin, cofilin, depactin, DnaseI, villin, fragmin, severin, capping protein,  $\beta$ -actinin and acumentin as set forth herein. Thus, the invention includes the use of a polypeptide comprising the amino acid sequence LKKTET (which may be within its primary amino acid sequence) and conservative variants thereof.

**[0026]** As used herein, the term "conservative variant" or grammatical variations thereof denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the replacement of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another, the replacement of a polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like.

**[0027]** T $\beta$ 4 has been localized to a number of tissue and cell types and thus, agents which stimulate the production of T $\beta$ 4 can be added to or comprise a composition to effect T $\beta$ 4 production from a tissue and/or a cell. Such agents

include members of the family of growth factors, such as insulin-like growth factor (IGF-1), platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor beta (TGF- $\beta$ ), basic fibroblast growth factor (bFGF), thymosin  $\alpha$ 1 (T $\alpha$ 1) and vascular endothelial growth factor (VEGF). More preferably, the agent is transforming growth factor beta (TGF- $\beta$ ) or other members of the TGF- $\beta$  superfamily.

[0028] Additionally, agents that assist or stimulate healing may be added to a composition along with T $\beta$ 4 or a T $\beta$ 4 isoform. Such agents include angiogenic agents, growth factors, agents that direct differentiation of cells. For example, and not by way of limitation, T $\beta$ 4 or a T $\beta$ 4 isoform alone or in combination can be added in combination with any one or more of the following agents: VEGF, KGF, FGF, PDGF, TGF $\beta$ , IGF-1, IGF-2, IL-1, prothymosin  $\alpha$  and thymosin  $\alpha$ 11 in an effective amount.

[0029] The actual dosage, formulation or composition that heals or prevents inflammation, damage and degeneration may depend on many factors, including the size and health of a subject. However, persons of ordinary skill in the art can use teachings describing the methods and techniques for determining clinical dosages as disclosed in PCT/US99/17282, *supra*, and the references cited therein, to determine the appropriate dosage to use.

[0030] In preferred embodiments, the concentration of the polypeptide is within a range of about 0.01-1 mole of the polypeptide per mole of the adhesive, more preferably within a range of about 0.1-0.5 mole of the polypeptide per mole of the adhesive, most preferably within a range of about 0.2-0.4 mole of the polypeptide per mole of the adhesive.

[0031] Suitable formulations may include T $\beta$ 4 or a T $\beta$ 4 isoform at a concentration within the range of about 0.001 - 10% by weight, within the range of about 0.01 - 0.1% by weight, or even about 0.05% by weight.

[0032] The invention includes use of antibodies which interact with T $\beta$ 4 peptide or functional fragments thereof. Antibodies which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the protein by methods well known to those skilled in the art as disclosed in PCT/US99/17282, *supra*. The term antibody as used in this invention is meant to include monoclonal and polyclonal antibodies.

[0033] In yet another embodiment, the invention provides a method of treating a subject by administering an effective amount of an agent which modulates T $\beta$ 4 gene expression. The term "modulate" refers to inhibition or suppression of T $\beta$ 4 expression when T $\beta$ 4 is over expressed, and induction of expression when T $\beta$ 4 is under expressed. The term "effective amount" means that amount of T $\beta$ 4 agent which is effective in modulating T $\beta$ 4 gene expression resulting in effective treatment. An agent which modulates T $\beta$ 4 or T $\beta$ 4 isoform gene expression may be a polynucleotide for example. The polynucleotide may be an antisense, a triplex agent, or a ribozyme. For example, an antisense directed to the structural gene region or to the promoter region of T $\beta$ 4 may be utilized.

[0034] In another embodiment, the invention provides a method for utilizing compounds that modulate T $\beta$ 4 activity. Compounds that affect T $\beta$ 4 activity (*e.g.*, antagonists and agonists) include peptides, peptidomimetics, polypeptides, chemical compounds, minerals such as zincs, and biological agents.

**[0035]** While not be bound to any particular theory, the present invention may promote healing or prevention of inflammation or damage by inducing terminal deoxynucleotidyl transferase (a non-template directed DNA polymerase), to decrease the levels of one or more inflammatory cytokines, or chemokines, and to  
5 act as a chemotactic factor for endothelial cells, and thereby promoting healing or preventing degenerative changes in tissue brought about by injury or other degenerative or environmental factors.

**[0036]** The invention is further illustrated by the following example, which is not to be construed as limiting.

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### Example

#### Proteins and Reagents

**[0037]** Human fibrinogen depleted of plasminogen, fibronectin and von Willebrand factor was purchased from Enzyme Research Laboratories (South  
15 Bend, IN). The recombinant  $\alpha$ C-fragment corresponding to the human fibrinogen  $\alpha$ C-domain (residues A $\alpha$ 221-610) and its truncated variants corresponding to the NH<sub>2</sub> and COOH-terminal halves (residues A $\alpha$ 221-391 and A $\alpha$ 392-610, respectively) were produced in E. coli using the pET20b expression vector. The recombinant  $\gamma$ -module comprising residues 148-411 of the human fibrinogen  $\gamma$   
20 chain was produced in E.coli using the same expression vector.

**[0038]** Bovine thrombin (1,000 NIH<sub>u</sub>/mg, aprotinin (4.4 TIU/mg), antirabbit IgG-horseradish conjugate and fluorescein isothiocyanate (FITC) were purchased from Sigma. Recombinant factor XIII was provided as a gift by Zymogenetics, Inc. (Seattle, WA). Synthetic thymosin  $\beta$ 4 was provided as a gift by Regenerx

Biopharmaceuticals, Inc. (Bethesda, MD). Anti-thymosin  $\beta$ 4 serum was prepared according to published methods.

#### Activation of factor XIII.

- 5 [0039] Factor XIII in 25 mM Tris buffer, pH 8.0, with .15 M NaCl (TBS), was activated either with thrombin or with  $\text{CaCl}_2$ ; the latter was made to avoid the presence of thrombin which could potentially activate fibrinogen. Thrombin-activated FFXIII [FXIIIa(THr)] was made by addition of bovine thrombin to final concentrations of 25 NIH u/ml and 2.5  $\text{CaCl}_2$  mM.  $\text{Ca}^{2+}$ -activated thrombin
- 10 [FXIIIa(Ca)] was made by addition of  $\text{CaCl}_2$  to final concentration of 50 mM. Final concentration of FXIII in both mixtures was 1.5 mg/ml; both mixture were incubated at room temperature for 10 min prior experiments.

#### Labeling of thymosin $\beta$ 4 with FITC

- 15 [0040] Fluorescence labeled thymosin  $\beta$ 4 was prepared by the reaction with fluorescein isothiocyanate (FITC). Thymosin  $\beta$ 4 was transferred in 0.1 M  $\text{NaHCO}_3$  buffer, pH 9.5, by gel-filtration on NAP5 Sephadex G-25 column (Amersham Biosciences) followed by addition of a 1.2 molar excess of FITC and incubation of the mixture at 37°C for 2 h in the dark. Non-reacted FITC was removed on NAP5
- 20 column. The degree of labeling determined spectrophotometrically as described was found to be 0.9 mole of FITC per mole of thymosin  $\beta$ 4.

#### Solid-phase Binding Assay

- [0041] The interaction between thymosin  $\beta$ 4 and fibrin(ogen) and its fragments
- 25 in the presence or absence of FXIIIa was studies by ELISA using plastic microliter plates. Wells of microliter plates were coated overnight at -4°C with fibrinogen

and fibrin at 10  $\mu\text{g/mL}$  or with the recombinant fragments of 20  $\mu\text{g/mL}$ , all in 0.1 M  $\text{NaHCO}_3$  buffer, pH 8.3. Fibrin was made by addition to the wells of a mixture containing 10  $\mu\text{g/mL}$  fibrinogen 1 NIH u/ml thrombin and 400 u/ml aprotinin, followed by overnight incubation at  $+4^\circ\text{C}$ . The wells were then blocked by  
5 incubation with Super Blocker (Pierce) at  $37^\circ\text{C}$  for 1 h. Following washing with TBS containing 0.05% Tween-20 (TBS-Tween), the indicated concentrations of thymosin  $\beta 4$ , FXIII, FXIIIa(Thr) and FXIIIa(Ca) were added to the wells and incubated for 2-2.5 h at  $37^\circ\text{C}$ . Bound (incorporated) thymosin  $\beta 4$  was detected by the reaction with rabbit anti-thymosin  $\beta 4$  serum and peroxidase-conjugated  
10 anti-rabbit IgG. A TMB Microwell Peroxidase Substrate was added to the wells, and the incorporated thymosin  $\beta 4$  was measured spectrophotometrically at 450 nm.

#### Incorporation of thymosin $\beta 4$ into fibrinogen and fibrin

15 **[0042]** Reactions of incorporation of FITC-labeled and unlabeled thymosin  $\beta 4$  into fibrinogen and fibrin were performed in Eppendorf tubes containing a mixture of fibrinogen at 3 mg/mL (9  $\mu\text{M}$ ) and thymosin  $\beta 4$  or FITC-labeled thymosin  $\beta 4$  at 150  $\mu\text{g/L}$  (30  $\mu\text{M}$ ) in 100  $\mu\text{L}$  TBS with 2.5 mM  $\text{CaCl}_2$ . The reactions were initiated by addition of FXIIIa(Ca) or FXIIIa(Thr) to final  
20 concentration of 30  $\mu\text{g/mL}$ . The final concentration of thrombin in the FXIIIa(Thr)-containing mixtures was made at 2.5 NIH u/mL, sufficient to rapidly form fibrin clot which was observed visually. The reactions with FITC-labeled thymosin  $\beta 4$  lasted for 4 hours at  $37^\circ\text{C}$  in the dark and were stopped by heat inactivation of the enzymes in boiling water for 5 min during fibrinogen and  
25 fibrin denatured and precipitated. The pellets were centrifuged and washed 3

times in TBS and then solubilized. The amounts of fibrin(ogen) and FITC-labeled thymosin  $\beta$ 4 in the solubilized pellet were determined spectrophotomertrically using absorption molar coefficients  $E_{280,1\%} = 15.0$  and  $\epsilon_{495} = 72,000 \text{ M}^{-1}\text{cm}^{-1}$ , respectively. To prepare samples with unlabeled thymosin  $\beta$ 4 for analysis by  
5 SDS-PAGE and Western blot the reaction mixtures at the indicated time were heat-inactivated as above and solubilized by addition of sample buffer (Invitrogen) containing SDS and reducing agent.

### Kinetic Analysis

10 **[0043]** To analyze kinetics of the incorporation of thymosin  $\beta$ 4 into different fibrin(ogen) fragments, they were immobilized onto the wells of microliter plates (as described above, except that the concentration of all fragments was 20  $\mu\text{g/mL}$ ) and incubated with several concentrations of thymosin  $\beta$ 4 in the presence of 10  $\mu\text{g/L}$  thrombin-activated factor XIIIa. The incubation mixtures  
15 were inhibited every 15 min during 1 hour of incubation by the addition of iodacetamide to final concentration 10 mM incorporated thymosin  $\beta$ 4 at each time point was detected with rabbit anti-thymosin  $\beta$ 4 serum as described above. The initial rates of the reaction of incorporation ( $V$ ) at different concentrations of thymosin  $\beta$ 4 were determined from the slopes of the reaction time course plots  
20 and expressed as tangent  $\alpha = A_{450}/t$  (min), where  $A_{450}$  represents absorbance at 450 nm in optical units (o.u) which is proportional to the amount of incorporated thymosin  $\beta$ 4. Apparent Michaelis constants,  $K_m$ , were obtained from Lineweaver-Burk plots,  $1/V$  (min/o.u.) versus  $1/[S](\mu\text{M}^{-1})$ , where  $[S]$  is concentration of thymosin  $\beta$ 4.

### Western Blot Analysis

[0044] Detection of thymosin  $\beta$ 4 incorporated into fibrin(ogen) and its fragments was performed as follows. The samples prepared as described above were electrophoresed and electrotransferred to a nitrocellulose membrane (Invitrogen) as described earlier. The membrane was blocked with a casein blocker for 1 hour and thymosin  $\beta$ 4 was detected by the reaction with rabbit anti-thymosin  $\beta$ 4 serum and peroxidase-conjugated anti-rabbit IgG. Visualization of the peroxidase-labeled protein bands was performed by the procedure recommended by the manufacturer using a supersignal west pico chemiluminescent substrate.

### ELISA-detected Incorporation of thymosin $\beta$ 4 into Fibrinogen and Fibrin

[0045] To test that factor XIIIa could mediate cross-linking of thymosin  $\beta$ 4 to fibrin(ogen), and to clarify the mechanism of such cross-linking we performed a direct study of the interaction of thymosin  $\beta$ 4 with fibrinogen and fibrin in the presence and absence of recombinant factor XIII. It should be noted that the recombinant factor comprises two  $\alpha$  subunits ( $\alpha_2$ ), in contrast to plasma factor XIII corresponds to the platelet form of factor XIII.

[0046] In ELISA experiments, when thymosin  $\beta$ 4 at 150  $\mu$ g/mL (30  $\mu$ M) was incubated with immobilized fibrinogen, only a low signal was observed in the absence of factor XIII as well as in the presence of non-activated factor XIII suggesting that the interaction between them is very weak, if any. When thymosin  $\beta$ 4 was incubated with immobilized fibrin in the absence or presence of non-activated factor XIIIa, which was activated by the addition of  $\text{CaCl}_2$  to avoid conversion of fibrinogen into fibrin in the wells, the signal substantially increased



suggesting that factor XIIIa mediates binding (incorporation) of thymosin  $\beta$ 4 into fibrinogen. A similar situation was observed with immobilized fibrin except that the level of the incorporation was higher than that into fibrinogen. The incorporation in both cases was dose-dependent. The incorporation onto fibrin  
5 was further increased when factor XIII was activated with thrombin instead of  $\text{Ca}^{2+}$ . Such differences could be due to different specific activities of these two factor XIIIa species. These results indicate that, activated XIII, similarly to tissue transglutaminase, mediates incorporation of thymosin  $\beta$ 4 into both fibrinogen and fibrin. They also suggest that there is no significant non-covalent interaction  
10 thymosin  $\beta$ 4 and both fibrinogen and fibrin.

Further analysis of the incorporation of thymosin  $\beta$ 4 into fibrinogen and fibrin

**[0047]** To further characterize factor XIIIa-mediated incorporation of thymosin  $\beta$ 4 into fibrin(ogen), a mixture was analyzed of thrombin, factor XIII, thymosin  $\beta$ 4  
15 and fibrin at different time points by immunoblotting. The mixture and the samples for analysis were prepared as described in Experimental Procedures. The samples were electrotransferred to a nitrocellulose membrane and probed with anti- thymosin  $\beta$ 4 serum. The results of immunoblotting indicate that factor XIIIa incorporates thymosin  $\beta$ 4 into fibrin covalently, like tissue  
20 transglutaminase, and that the amount of the incorporated (cross-linked) thymosin  $\beta$ 4 seems to reach saturation after 4 hours. This time was selected to evaluate the degree of the incorporation. For this purpose thymosin  $\beta$ 4 was labeled with a FITC chromophore group which enabled its direct measurement in fibrinogen/ thymosin  $\beta$ 4 and fibrin/ thymosin  $\beta$ 4 mixtures. Such modification  
25 did not influence its incorporation into either fibrinogen or fibrin based on the

pattern of incorporation revealed by Western blot analysis. A similar mixture as above but with FITC-labeled thymosin  $\beta 4$  was incubated for 4 hours after which the degree of incorporation was estimated based on the spectrophotometrically determined amounts of fibrin(ogen) and incorporated FITC- thymosin  $\beta 4$  in each sample. The results revealed that at the selected conditions, which include physiological concentration of fibrinogen (9  $\mu$ M), factor XIIIa incorporated a substantial amount of FITC- thymosin  $\beta 4$ , about 0.2 and 0.4 moles per mole of fibrinogen and fibrin, respectively.

#### Incorporation of thymosin $\beta 4$ into individual fibrin(ogen) chains

**[0048]** To establish which of the three fibrin(ogen) chains are involved in cross-linking with thymosin  $\beta 4$ , we analyzed the time course of factor XIIIa-mediated cross-linking of fibrinogen and fibrin in the presence and absence of thymosin  $\beta 4$  by SDS-PAGE and Western blot. It is well known that in fibrin factor XIIIa cross-links rapidly the COOH-terminal portions of the  $\gamma$  chains to produce  $\gamma$ - $\gamma$  dimers followed by cross-linking of the  $\alpha$  chains to form  $\alpha$ - $\alpha$  dimers, trimers, and  $\alpha$ -polymers; fibrinogen is cross-linked in a similar way but at a slower rate. When analyzed by SDS-APGE in reducing conditions, the bands corresponding to the individual polypeptide chains of fibrinogen and fibrin,  $A\alpha$ ,  $B\beta$ ,  $\gamma$  and  $\alpha$ ,  $\beta$ ,  $\gamma$ , respectively, were well resolved. Incubation of fibrinogen with factor XIIIa resulted in progressive depletion of the band corresponding to the  $\gamma$ - $\gamma$  dimers and the  $A\alpha$ - $A\alpha$  dimers and trimers; the appearance of some material at the start which most probably corresponds to the  $A\alpha$  polymers was also observed. When fibrinogen was incubated with factor XIIIa in the presence of thymosin  $\beta 4$ , no substantial difference in the intensity of the bands corresponding to the

individual chains and their cross-linked variants was found. Similar results were obtained with fibrin except that the cross-linking of its  $\alpha$  and  $\gamma$  chains occurred more rapidly, as expected, and the amount of the material at the start was higher. Subsequent Western blot experiments revealed that after 30 min of incubation substantial amount of thymosin  $\beta 4$  was incorporated into fibrinogen  $A\alpha$  chain and that after 150 min of incubation some thymosin  $\beta 4$  was also incorporated into the  $A\alpha$ - $A\alpha$  dimer. The incorporation of thymosin  $\beta 4$  into fibrin  $\alpha$  chain and the  $\alpha$ - $\alpha$  dimer was much more rapid and after 150 min of incubation material of thymosin  $\beta 4$  was also observed in higher molecular mass forms of the  $\alpha$  chain ( $\alpha$  polymers). These results indicate that the fibrinogen  $A\alpha$  and fibrin  $\alpha$  chains contain the major sites for covalent incorporation of thymosin  $\beta 4$ . At the same time the appearance after 150 min of incubation of a low intensity band with the mobility between that of the  $\gamma$ - $\gamma$  and  $\alpha$ - $\alpha$  dimers suggests that thymosin  $\beta 4$  could also be incorporated into the fibrin  $\gamma$  chains ( $\gamma$ - $\gamma$  dimer). Alternatively, this band may correspond to a proteolytically truncated variant of the  $\alpha$ - $\alpha$  dimer.

#### Incorporation of thymosin $\beta 4$ into recombinant fibrin(ogen) fragments

**[0049]** It is well established that the COOH-terminal proteins of the fibrinogen  $A\alpha$  and  $\gamma$  chains forming the  $\alpha C$  domain and  $\gamma$ -module contain reactive Gln and Lys residues which are cross-linked by factor XIIIa in fibrin and therefore could potentially be involved in cross-linking with thymosin  $\beta 4$ . To test this and to further localize the cross-linking sites for thymosin  $\beta 4$  in fibrin(ogen), was analyzed incorporation of thymosin  $\beta 4$  into the recombinant  $\gamma$ -module (residues  $\gamma 148$ -411) and the  $\alpha C$ -domain ( $A\alpha 221$ -391 and  $A\alpha 392$ -610 sub-fragments, by SDS-PAGE and Western blotting. Incubation of the  $\alpha C$ -domain and the  $\gamma$ -module

with factor XIIIa in the presence of thymosin  $\beta$ 4 resulted in effective cross-linking and appearance of their appearance of their higher molecular mass forms, dimers, trimers and oligomers. At the same time, the cross-linking of the A $\alpha$ 221-391 and A $\alpha$ 392-610 sub-fragments, which contain mainly acceptor Gln and donor Lys residues, respectively, was much less effective. When the samples were electrotransferred to nitrocellulose membrane and probed with anti-thymosin  $\beta$ 4 serum, substantial amounts of thymosin  $\beta$ 4 were detected in the  $\alpha$ C-domain, the  $\gamma$ -module and their higher molecular mass variants, dimers, trimers and oligomers. The incorporation into the A $\alpha$ 392-610 sub-fragment monomer and oligomers was also substantial while only very small amount of thymosin  $\beta$ 4 was detected in the A $\alpha$ 221-391 oligomers. These results suggest that thymosin  $\beta$ 4 could be cross-linked to both the  $\alpha$ C-domain and the  $\gamma$ -module, and that the reactive Lys residues of the A $\alpha$ 392-610 region of the former are involved in the cross-linking.

**[0050]** The above observations were confirmed by ELISA. When thymosin  $\beta$ 4 was incubated with the immobilized  $\gamma$ -module or the  $\alpha$ C-domain variants in the presence of factor XIIIa, it was incorporated effectively into the  $\gamma$ -module and into the  $\alpha$ C-domain and the A $\alpha$ 392-610 sub-fragment while the incorporation into A $\alpha$ 221-391 was very low. It should be noted that the incorporation of the  $\gamma$ -module was almost twice lower than that of the  $\alpha$ C-domain variants at all concentration studied. When thymosin  $\beta$ 4 was incubated with the same immobilized species in the presence of non-activated factor XIII or without it, the incorporation was very low in all cases. This suggests that, as in the case with

fibrinogen and fibrin, there is no significant non-covalent interaction between thymosin  $\beta 4$  and the recombinant fragments.

**[0051]** It was previously shown that factor XIIIa cross-linking of the  $\gamma$  chains of fibrin exhibits apparent Michaelis behavior. Assuming that factor XIIIa behaves  
5 as a Michaelis enzyme when cross-linking thymosin  $\beta 4$  to the immobilized  $\gamma$ -module and  $\alpha$ C-domain variants one could determine the kinetic parameters of such cross-linking. The analysis of the kinetic data revealed the following values of apparent Michaelis constants ( $K_m$ ) for the reaction of incorporation,  $183 \pm 29$   $\mu$ M for the incorporation of thymosin  $\beta 4$  into the  $\gamma$ -module, and  $17.6 \pm 2.5$   $\mu$ M  
10 and  $8.6 \pm 3.7$   $\mu$ M for that into the  $\alpha$ C-domain and its A $\alpha$ 392-610 sub-fragment, respectively. The much higher  $K_m$  value for the  $\gamma$ -module than those for the  $\alpha$ C-domain and its sub-fragment indicates that the cross-linking of thymosin  $\beta 4$  to the  $\alpha$ C-domain variants is much more efficient. In this connection, the  $K_m$  for the A $\alpha$ 392-610 fragment is comparable to the  $K_m = 6.2$   $\mu$ M determined previously for  
15 the factor XIIIa-mediated  $\gamma$ - $\gamma$  cross-linking. The two-fold difference in the  $K_m$  values for the  $\alpha$ C-domain and the A $\alpha$ 392-610 sub-fragment could be explained by competition between reactive Gln residues of thymosin  $\beta 4$  and the A $\alpha$ 392-610 region, i.e., between  $\alpha$ C-to- $\alpha$ C and thymosin  $\beta 4$ -to- $\alpha$ C cross-linking. In agreement, the double-reciprocal plot for the  $\alpha$ C-domain and the A $\alpha$ 3962-610  
20 sub-fragment exhibits a pattern characteristic for competitive inhibition.

**[0052]** Altogether, the results indicated that factor XIIIa effectively cross-links thymosin  $\beta 4$  to the COOH-terminal portion of the isolated  $\alpha$ C-domain including residues A $\alpha$ 3962-610, that the incorporation into the isolated  $\gamma$ -module is less

effective, and that in fibrinogen or fibrin the incorporation occurs mainly in the  $\alpha$ C-domains.

**[0053]** Fibrin(ogen) plays an important role in wound healing through interactions with physiologically active proteins and cell receptors. Particularly, the fibrin matrix stimulates an inflammatory response and capillary tube formation by endothelial cells (angiogenesis), which are essential steps in the wound healing process, through interaction with the leukocyte integrin Mac-1 and endothelial cell receptor VE-cadherin, respectively. It also interacts with high affinity with basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) providing co-localization of these potent stimulators of angiogenesis at sites of fibrin deposition and their contribution to wound healing. Fibrin can also retain at insulin-like growth factor binding protein-3 (IGFBP-3), which forms a complex with IGF-1. Thymosin  $\beta_4$ , a potent angiogenic and wound healing factor, can also be incorporated into fibrin by tissue transglutaminase and apparently further increase the wound healing potential of fibrin matrix.

**[0054]** Although all transglutaminases catalyze the same reaction, formation of covalent  $\gamma$ -glutamyl- $\epsilon$ -lysyl isopeptide bonds between reactive Gln and Lys residues, their specificity towards substrates may differ. For example, while factor XIIIa, a plasma transglutaminase, specifically cross-links in fibrin the  $\gamma$  and  $\alpha$  chains resulting in the  $\gamma$ - $\gamma$  dimers and  $\alpha$ -polymers, respectively, tissue transglutaminase is less specific and can also generate  $\alpha$ - $\gamma$  chains cross-links. The cross-linking patterns for the serine protease inhibitor (serpin), PAI-2, to fibrin(ogen) were also found to be different for tissue transglutaminase and factor XIIIa. It was originally shown that thymosin  $\beta_4$  is incorporated into fibrin by guinea pig liver tissue transglutaminase; its incorporation into fibrin by factor

XIIIa was hypothesized based on the facts that thrombin-activated platelets co-release factor XIII and thymosin  $\beta_4$  and that the latter becomes cross-linked to fibrin. In this study it was demonstrated directly that thymosin  $\beta_4$  is incorporated by factor XIIIa to both fibrinogen and fibrin. Furthermore, it was found that the degree of the incorporation is rather high, 0.2 and 0.4 mole of thymosin  $\beta_4$  per mole of fibrinogen and fibrin, respectively. Since concentration of fibrinogen in plasma is about 9  $\mu$ M, local concentration of fibrin at places of fibrin deposition should be much higher. Taking into account that thymosin  $\beta_4$  exhibits its proangiogenic activity at 0.1 nM-1  $\mu$ M, such degree of incorporation is obviously physiologically significant and should be sufficient to increase the wound healing potential of fibrin clot.

**[0055]** It is known that factor XIIIa incorporates into fibrin a number of plasma proteins,  $\alpha_2$ -antiplasmin, PAI-2, fibronectin, thrombospondin, and von Willebrand factor. The mechanism of incorporation is established only for some of them. For example, fibronectin binds to the fibrin  $\alpha$ C-domains non-covalently with high affinity prior to covalent cross-linking with factor XIIIa; the recognition sites and the reactive Gln and Lys residues in each protein are located in different regions providing proper orientation of the cross-linking sites. In addition, factor XIIIa interacts with the  $\alpha$ C-domains further increasing the specificity of the reaction. To test whether non-covalent binding of thymosin  $\beta_4$  precedes its cross-linking to fibrin, its interaction was studied with immobilized fibrinogen and fibrin in the presence and absence of non-activated factor XIII. In contrast to other proangiogenic factors such as bFGF and VEGF, which exhibit high affinity to fibrin, no noticeable non-covalent interaction was observed with thymosin  $\beta_4$  in all cases. The incorporation was observed only in the presence of activated factor

XIIIa suggesting that the covalent cross-linking may be the only mechanism to retain thymosin  $\beta_4$  in fibrin clot.

[0056] The results clearly indicate that although thymosin  $\beta_4$  could be incorporated by factor XIIIa into the isolated  $\gamma$ -module and the  $\alpha$ C-domain variants, in fibrin(ogen) it is cross-linked mainly to the  $\alpha$ C-domains, namely to their Aa392-610 regions. The analysis of distribution of the identified reactive Lys and Gln residues in thymosin  $\beta_4$  and fibrin(ogen) provides a reasonable explanation for this finding. Thymosin  $\beta_4$  contains a reactive amine donor, Lys38, and two amine receptors, Gln23 and Gln36, which could be involved in the cross-linking reaction with other proteins. There are only two reactive residues in the  $\gamma$  chain involved in the intermolecular  $\gamma$ - $\gamma$  cross-linking, Gln398 (or Gln399) and Lys406, both located in the  $\gamma$ -module. When the isolated  $\gamma$ -module was treated with factor XIIIa, the cross-linking seemed to occur randomly resulting in dimers, trimers/oligomers; thymosin  $\beta_4$  was incorporated in all these species. In fibrin, these regions are aligned by the DD:E interactions in an antiparallel manner facilitating cross-linking between Gln398/399 of one chain and Lys406 of another to form  $\gamma$ - $\gamma$  dimers. The efficiency of this cross-linking reaction is much higher than that between these residues and thymosin  $\beta_4$ , and therefore it is not surprising that little or no incorporation of thymosin  $\beta_4$  into the fibrin  $\gamma$  chains was observed in this study.

[0057] In contrast to the  $\gamma$  chain, the  $A\alpha$  chain contains multiple reactive glutamine and lysine residues. The following residues were found to be involved in the cross-linking between the  $\alpha$  chains in fibrin or the recombinant  $\alpha$ C-domains, Gln221, 237, 328 and 366, and Lys508, 539, 556, 580 and 601. The  $A\alpha$  chain Lys303 was shown to serve as amine donor in factor XIIIa-mediated



cross-linking of the serpin  $\alpha_2$ -antiplasmin to fibrin(ogen). This Lys is not reactive towards another serpin, PAI-2, which is cross-linked by tissue transglutaminase and factor XIIIa through other A $\alpha$  chain lysine residues, 148, 176, 183, 230, 413 and 457. The study with a synthetic peptide mimicking the cross-linking region of  $\alpha_2$ -antiplasmin revealed that it is incorporated into fibrin  $\alpha$  chain through 12 reactive lysine residues, Lys418, 448, 508, 539, 556 and 580, which accounted for 78% of the total activity, and less reactive Lys208, Lys219 and/or 224, Lys427, 429, 601 and 606. At least 10 lysine residues within fibrin(ogen) A $\alpha$ 368-610 region were implicated in cross-linking reactions with fibronectin. The above analysis indicates that most of the identified reactive residues in fibrin are located in its  $\alpha$ C-domains, that the 392-610 region of the  $\alpha$ C-domain, to which thymosin  $\beta_4$  is a preferentially cross-linked, contains at least 11 reactive Lys residues, and that among these residues only half is utilized in the  $\alpha$ - $\alpha$  cross-linking. It also suggests that although thymosin  $\beta_4$  could compete for reactive lysine residues involved in the  $\alpha$ - $\alpha$  cross-linking, its cross-linking to the  $\alpha$ C-domains may occur independently of their intermolecular  $\alpha$ - $\alpha$  cross-linking providing its efficient incorporation into fibrin. Thus the reactive lysine residues of the  $\alpha$ C-domains not only serve for the  $\alpha$ - $\alpha$  cross-linking but also simultaneously accommodate physiologically active proteins, including thymosin  $\beta_4$ , which could modulate properties of fibrin matrix contributing to wound healing and other physiological and pathological processes.

**[0058]** Fibrinogen polymerizes in a controllable fashion to make a clot which easily adheres to different cells and is non-immunogenic and biodegradable. These make it an ideal hemostatic and bioadhesive (fibrin sealant) that has been used increasingly in numerous surgical applications as an hemostatic agent for

the arrest of bleeding, and to assist tissue sealing and wound healing. The use of fibrin sealants in wound healing and other therapies can be enhanced by including bioactive agents. For example, it was shown in cellular and animal models that fibrin can serve as a vehicle for localized delivery of antibiotics and growth factors. While antibiotics encapsulated by fibrin are released slowly due to low solubility, the retention of growth factors in fibrin sealants was achieved through their high affinity interaction with fibrin, or through their direct covalent cross-linking to it. The ability of thymosin  $\beta_4$  to be incorporated into fibrin(ogen) by cross-linking with factor XIIIa could be used for its immobilization on fibrin sealants. This study demonstrates high efficiency of such incorporation into both fibrinogen and fibrin, supporting this approach.

**[0059]** In summary, experimental studies confirm that thymosin  $\beta_4$ , a bioactive peptide, could be incorporated into fibrin by covalently cross-linking with factor XIIIa, demonstrated high efficiency of its incorporation into both fibrinogen and fibrin at physiological concentrations of the components, and localized the incorporation sites within the A $\alpha$ 392-610 region of the fibrin(ogen)  $\alpha$ C-domains. Experimental data supports incorporation of physiologically significant amounts of thymosin  $\beta_4$  into fibrin sealants for delivery to places of wound healing.

**[0060]** Tissue transglutaminase and presumably plasma transglutaminase, factor XIIIa, can covalently incorporate into fibrin(ogen) a physiologically active peptide, thymosin  $\beta_4$ . To clarify the mechanism of this incorporation interaction was studied of thymosin  $\beta_4$  with fibrinogen, fibrin, and their recombinant fragments, the  $\gamma$ -module ( $\gamma$  chain residues 148-411), and the  $\alpha$ C-domain (A $\alpha$  chain residues 221-610) and its truncated variants by immunoblot and ELISA. No significant non-covalent interaction between them was detected in the

absence of activated factor XIII while in its presence thymosin  $\beta_4$  was effectively incorporated into fibrin and to a lesser extent into fibrinogen. The incorporation at physiological concentrations of fibrin(ogen) and factor XIII was significant with molar incorporation ratios of thymosin  $\beta_4$  to fibrinogen and fibrin of 0.2 and 0.4, respectively. Further experiments revealed that although activated factor XIII incorporates thymosin  $\beta_4$  into the isolated  $\gamma$ -module and  $\alpha$ C-domain, in fibrin the latter serves as the major incorporation site. This site was further localized to the COOH-terminal portion of the  $\alpha$ C-domain including residues 392-610.